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14. ABSTRACT

The purpose of this proposal is to test the hypothesis that acetylation of PGC-1alpha by the Acetyl Transferase GCN5 associated proteins, Pc3 and WDR18, is a key regulatory modification that controls hepatic glucose production. This investigation has a define scope to specifically test how these proteins control the acetylation status of PGC-1alpha and what is the functional effect in blood glucose levels. The major findings of this Research Technical Report are that we have generated specific siRNAs that specifically knock-down Pc3 and WDR18 and control PGC-1alpha transcriptional function. Moreover, we have generated PGC-1alpha acetylation mutants, surprisingly GCN5 still repress these mutant indicating that other sites are also involved. The significance of these results are that we have identified two proteins, Pc3 and WDR18, that control PGC-1alpha activity and that previous acetylation sites identified in PGC-1alpha are no responsible for GCN5-mediated repression of PGC-1alpha. This leads to further effort to identify the specific sites on PGC-1alpha that are mediating the effects of GCN5 to control hepatic glucose production.

15. SUBJECT TERMS
Blood glucose; Stress conditions; Gene regulation

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Introduction

Homeostatic mechanisms in mammals, including humans, function to maintain blood glucose levels within a narrow range on response to hormones and nutrients. For example, high stress and intense exercise conditions combined with food deprivation make soldiers very vulnerable to changes in blood glucose levels. Glucose homeostasis is highly disregulated in metabolic diseases such as obesity and diabetes which have high incidence in the US population. We study a biochemical process that controls blood glucose levels through control of hepatic glucose synthesis. This regulatory control is achieved by a chemical modification –acetylation- of the PGC-1α metabolic transcriptional coactivator [1 2]. The main purpose and scope of this Research Proposal is to decipher how two proteins, Pc3 and WDR18, that control the enzymatic activity of the PGC-1α GCN5 Acetyl Transferase regulate PGC-1α acetylation and its effects on glucose metabolism. We are using biochemical and physiological approaches, both in cell culture and mice models, to precisely identify the key acetylation sites on PGC-1α that are required and sufficient to modulate blood glucose levels. These findings have strong implications for the basic pathways of energy homeostasis, diabetes and metabolic diseases and will certainly benefit performance of personnel in the army that work in conditions of stress.

Body

In this body section of this Research Technical Report, we will describe in detail the experimental data and interpretations of the results obtained in this first year of the award. As previously stated in the SOW we have performed experiments that were proposed in Tasks 1 and 3. We have accomplished the goals that were originally stated and based on the results we are proposing to continue the Tasks that are detailed in the SOW that is enclosed in this Research Technical Report.

Task 1. Analysis of two novel proteins in the GCN5 complex (Pc3 and WDR18) that strongly repress PGC-1α glucose production function. Importantly, WDR18 is regulated by nutritional status in diabetic models. (Months 1-24).

• Genetic Approaches using siRNAs. Design and test specific siRNAs for Pc3 and WDR-18 proteins of the GCN5 complex. (Months 1-12).

1. Generation of specific siRNAs for Pc3 (Pc3 according to the new nomenclature has been re-named CCDC101).

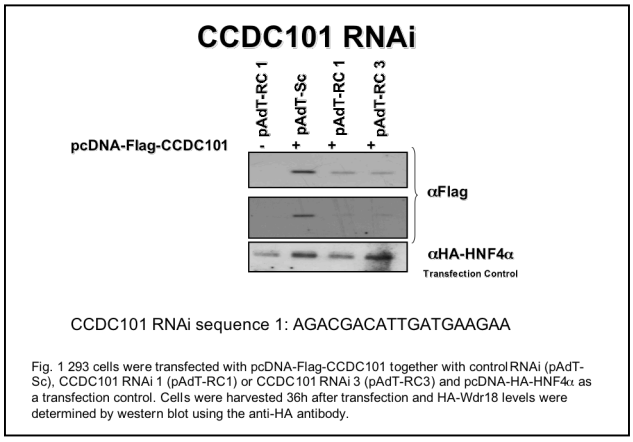

Fig. 1 293 cells were transfected with pcDNA-Flag-CCDC101 together with control RNAi (pAdT-Sc), CCDC101 RNAi 1 (pAdT-RC1) or CCDC101 RNAi 3 (pAdT-RC3) and pcDNA-HA-HNF4α as a transfection control. Cells were harvested 36h after transfection and HA-Wdr18 levels were determined by western blot using the anti-HA antibody.

In order to study the effects of CCDC101 in PGC-1α glucose production activity, we generated an specific siRNA to knock dowm CCDC101 protein levels in cells. To design the siRNA, we routinely use the program offered by Dharmacon that is based in different established rules and it provides the more likely siRNAs that will effectively decreased

levels of the targeted protein of interest. To test whether the designed specific siRNAs decreased CCDC101 protein levels, we transfected cells with a plasmid encoding CCDC101 to express the protein and with a plasmid encoding different siRNAs designed against the CCDC101 mRNA sequence (note the sequence of the siRNA sequence that has been selected in Fig. 1). As shown in Fig. 1 the designed siRNAs specifically knock down CCDC101 protein by a decrease that was estimated to be more that 80%. Based on this experiment, we conclude that CCDC101 siRNA has been validated and can be used to test the function of CCDC101 to modulate PGC-1α glucose metabolic effects.

2. Generation of specific siRNAs for WDR-18.

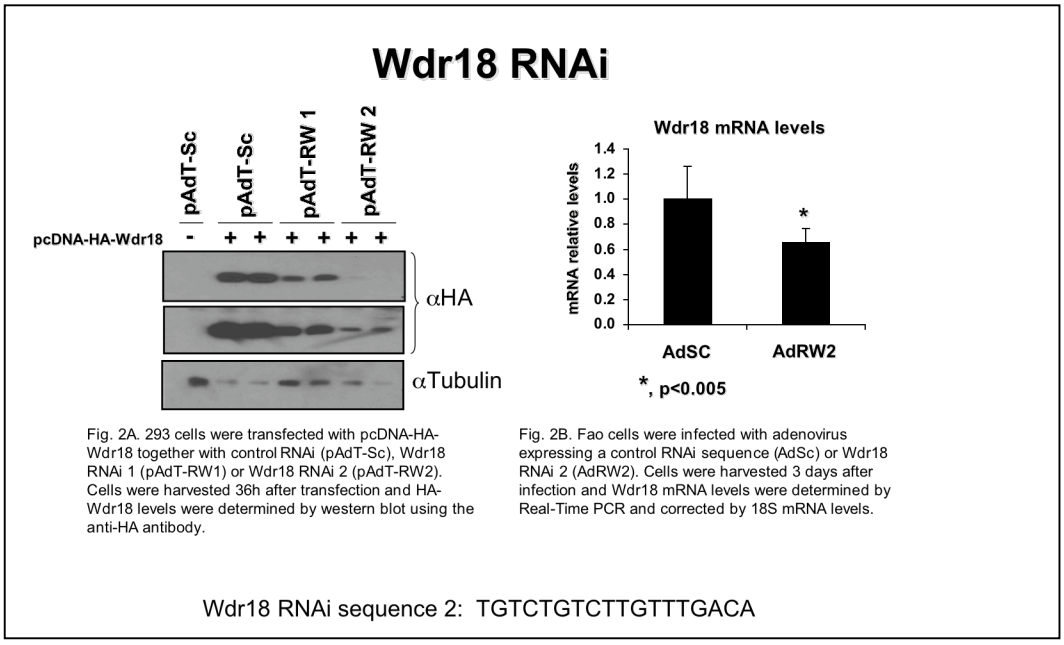

Fig. 2A. 293 cells were transfected with pcDNA-HA-Wdr18 together with control RNAi (pAdT-Sc), Wdr18 RNAi 1 (pAdT-RW1) or Wdr18 RNAi 2 (pAdT-RW2). Cells were harvested 36h after transfection and HA-Wdr18 levels were determined by western blot using the anti-HA antibody.

Fig. 2B. Fao cells were infected with adenovirus expressing a control RNAi sequence (AdSc) or Wdr18 RNAi 2 (AdRW2). Cells were harvested 3 days after infection and Wdr18 mRNA levels were determined by Real-Time PCR and corrected by 18S mRNA levels.

Wdr18 RNAi sequence 2: TGTCTGTCTTGTTTGACA

We also followed the same strategy -decribed above for CCDC101- to design and generate an specific siRNA for WDR-18 –see Fig. 2 legend for the specific nucleotide sequence-. Fig. 2A shows that designed WDR-18 siRNA efficiently knock down WDR18 protein expression in cells. We used tubulin as a control and was not affected by transfection of the different siRNAS. Based on the effects of the two siRNAs tested we decided to use the sequence number 2. In this case the percentage of knock down was almost 100% in the conditions that we tested. In order to further test this specific shRNA on endogenous Wdr18, we generated an adenoviruses that encodes the siRNA for WDR-18 and infected hepatic cells. As shown in Fig. 2B, infection with adenovirus for WDR18 siRNA (we checked infection by detecting GFP expression in cells) caused approximately a 50% reduction in endogenous mRNA encoding for this protein. Note that we could not test endogenous protein since antibodies are not currently available. Taken together these experiments, we conclude that WDR-18 siRNA has been validated and can be used to test the function of WDR-18 to modulate PGC-1α glucose metabolic effects.

• *Test of different siRNAs (for CCDC101 and WDR-18) on PGC-1α function. Transcriptional analysis using luciferase reporter systems. (Months 12-24).*

1. Effects of CCDC101 on PGC-1α transcriptional activity.

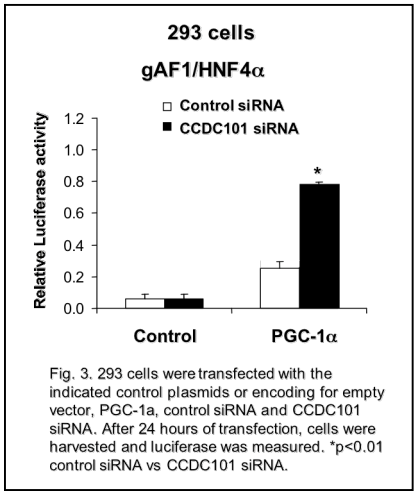

Fig. 3. 293 cells were transfected with the indicated control plasmids or encoding for empty vector, PGC-1a, control siRNA and CCDC101 siRNA. After 24 hours of transfection, cells were harvested and luciferase was measured. *p<0.01 control siRNA vs CCDC101 siRNA.

The fact that PGC-1α is a potent transcriptional coactivator allows to use luciferase transcriptional based assays to test its activity [3 4]. These type of assays become a very useful to study different signaling pathways or proteins that modulate PGC-1α function. Since PGC-1α coactivates different transcription factors depending on the metabolic program, we have used the HNF4α system since it is a key transcription factor that is required for PGC-1α to activate gluconeogenic gene expression [4].. The main objective of this task is whether CCDC101, as a protein identified in the GCN5 complex [2], can regulate PGC-1α acetylation and as a consequence to effect its transcriptional gluconeogenic gene expression activity. We therefore analyzed the effects of knocking down CCDC101 on the transcriptional activity of the HNF4α/PGC-1α system. As shown in Fig. 3, knock down of CCDC101 did not have any effect on HNF4α activity measured by luciferase units. As expected, PGC-1α increased this activity by 10 fold. Notably, co-expression of PGC-1α plus siRNA CCDC101 resulted in a 3 fold increased compared to PGC-1α alone. Based on these results we conclude that CCDC101 regulate the transcriptional activity of PGC-1a on the HNF4a system, a transcription factor that is required for PGC-1α to induce hepatic gluconeogenic gene expression.

2. Effects of WDR-18 on PGC-1α transcriptional activity.

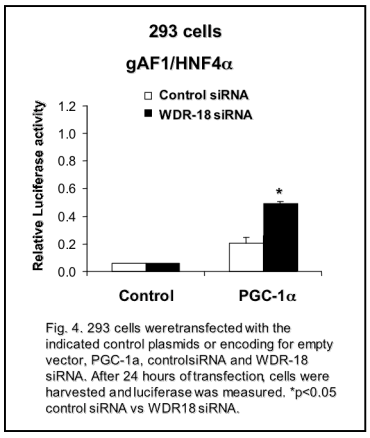

Fig. 4. 293 cells were transfected with the indicated control plasmids or encoding for empty vector, PGC-1a, control siRNA and WDR-18 siRNA. After 24 hours of transfection, cells were harvested and luciferase was measured. *p<0.05 control siRNA vs WDR18 siRNA.

We have also used the same strategy to investigate whether endogenous WDR-18 can regulate the transcriptional activity of PGC-1α on the HNF4α system. We therefore used the same siRNA described in the previous section. As shown in Fig. 4, knock down of WDR18 caused a 2.5 fold increase in the transcriptional activity of HNF4α/PGC-1α. These results indicate that endogenous WDR-18 regulates the transcriptional function of this nuclear receptor-coactivator system. Experiments proposed in the Statement of Work will further elucidate whether these effects on based-luciferase promoters can also translate to endogenous expression of genes both in cultured hepatocytes as well as in mice.

Task 3. Identification of the important functional acetylated lysines involved in PGC-1α repression. (Months 1-24).

• *Generation of PGC-1α lysine mutants. Site-directed mutagenesis methodology. (Months 1-6).*

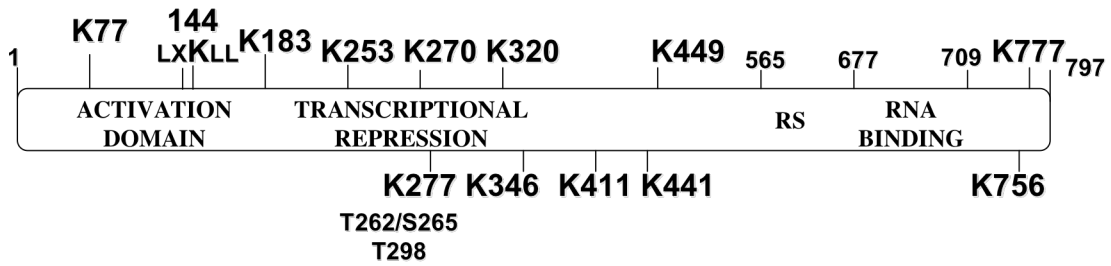

Figure 5. PGC-1α acetylated lysines. Mapping of the lysine acetylated residues was performed by mass spectrometry. Flag-PGC-1α was immunoprecipitated from 293 cells treated with 10mM nicotinamide. Protein from colloidal Coomassie stained bands was in-gel reduced, alkylated, and subsequently digested with either trypsin, with trypsin followed by chymotrypsin, or with endoproteinase Arg-C followed by chymotrypsin . Peptides were analyzed by nanoscale-microcapillary reversed phase liquid chromatography tandem mass spectrometry (LC-MS/MS) using a hand-pulled microcapillary column packed with reversed phase material and either an LCQ-Deca XP ion trap, an LTQ linear quadrupole ion trap, or an LTQ-FT linear quadrupole ion trap/Fourier transform mass spectrometer (Thermo Electron). The MS/MS data were searched against the Flag-PGC-1 α sequence using the SEQUEST algorithm allowing for the detection of oxidized methionines, carboxyamidomethylated cysteines , and acetylated lysines. High-scoring peptide matches [minimal cross-correlation scores(*Xcorr*) of 2.o and 3.o for ions with a charge state of 2+, and 3+, respectively; minimal delta-correlation score (*δCn*) of 0.1] with acetylation on lysine residues were validated manually.

We have previously found that PGC-1α is strongly deacetylated in the liver of fasted mice and this correlates with the activated gluconeogenic function of PGC-1α [1]. In collaboration with Steve Gygi (Department of Cell Biology, Harvard Medical School) we mapped 13 acetylation lysine sites on PGC-1α (Fig. 5). These acetylation sites are distributed along the different PGC-1α functional domains, but the function of each of the specific sites is unknown. Although it is likely that these sites are directly acetylated by GCN5, we first analyzed whether a PGC-1α mutant in which these 13 lysines have been mutated to arginine (R13). To generate this mutant we used site-directed mutagenesis and each mutation was further confirmed by DNA sequencing analysis. We therefore performed experiments to investigate whether the PGC-1α R13 allele was acetylated by GCN5. To our surprise the R13 mutant was still acetylated by GCN5 to a similar extent as the wild type (Fig. 6). Since this was an unexpected result, we are pursuing to determine what additional PGC-1α lysine sites are acetylated in the R13 mutant. This includes an ongoing collaboration with Steve Gygi, to generate a mutant (lysines to arginines, since this mimics the positive amino acid charge) that is no longer acetylated by GCN5. In this regard, we will use the same assay to determine whether GCN5 acetylates the generated mutant. Please note that this is not a change for the future work and constitutes part of the original Statement of Work.

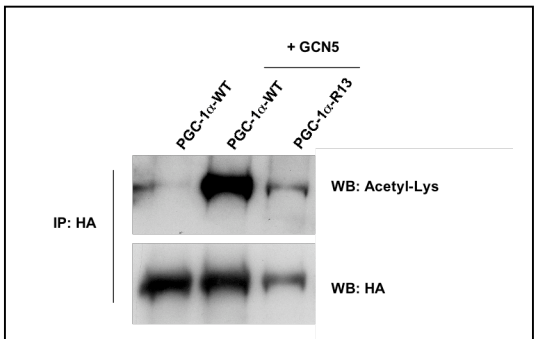

Fig. 6. PGC-1α R13 mutants is still acetylated by GCN5. FAO cells were infected with adenoviruses expressing PGC-1α wild type or R13 mutant (13 lysines detailed in Fig.5 were mutated to arginine. After 2 days of infection cells were harvested and PGC-1α was immunoprecipitated with an HA antibody and western blot analysis was performed with the indicated antibodies.

There is a possible caveat of generating such a mutant. It is still conceivable that the endogenous sites that GCN5 acetylates are the

ones that have been mapped, however when mutated it acetylates other lysines. Two strategies will be followed to determine: (i) to identify by mass spectrometry the lysines that are acetylated by GCN5 both in the wild-type as well as the R13 mutant, (ii) To functionally analyze the generated mutants on transcriptional activity using luciferase-based assays –see next section-. Overall, we are in an excellent position to generate mutants that are no longer acetylated by GCN5 and test the function of these mutants on the PGC-1α gluconeogenic activity.

> • *Transcriptional analysis of PGC-1α acetylated mutants and regulation by GCN5 associated proteins CCDC101 and WDR-18. Analysis using luciferase reporter systems (Months 6-24).*

As mentioned above although the R13 PGC-1α acetylation mutant was still acetylated by GCN5, it might be conceivable that these sites were important for GCN5-mediated repression of PGC-1α function. To test this possibility, we analyze the transcriptional activity of the R13 mutant in the presence or absence of GCN5. Cells were transfected with plasmids encoding for PGC-1α wild-type, R13 mutant and GCN5. As shown in Fig. 6, as expected GCN5 repressed the transcriptional activity of PGC-1α on the HNF4α-luciferase based system. Consistent with the fact that GCN5 acetylated the R13 mutant, transfection of R13 resulted in a strong HNF4α-dependent coactivation, however when GCN5 was transfected caused a similar repression compared to PGC-1α wild-type. Taken together, these results strongly indicate that the 13 lysines (which are mutated to arginine in the R13 allele) are not required for GCN5-mediated repression. This result, together with the fact that the R13 mutant is still acetylated indicate that other lysine are involved in this repression.

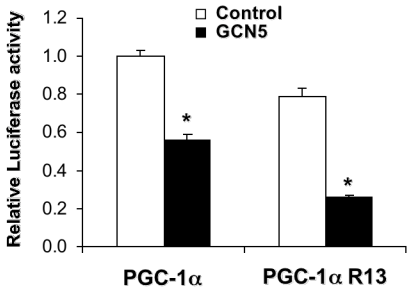

Fig. 7. PGC-1α R13 mutants is still repressed by GCN5. 293 cells were transfected with plasmids encoding PGC-1α wild type or R13 mutant (13 lysines detailed in Fig.5 were mutated to arginine), GCN5, HNF4α and gAF1-luciferase (A DNA binding site for HNF4α in the PEPCK promoter) as well as control plasmids. After 2 days of transfection cells were harvested and luciferase activity was measured. *p<0.01 PGC-1α vs PGC-1α+GCN5.

In the next 12 months this task will be expanded (as originally stated in the Statement of Work) to analyze additional lysines that are key to GCN5-mediated repression of the transcriptional coactivator PGC-1α. At this point to identify these new acetylated lysines is essential to fully accomplish the other tasks. Therefore, our major efforts will be devoted to perform the proposed experiments.

Key Research Accomplishments

In this first year, we have accomplished the tasks (Tasks 1 and 3) that were originally proposed in the application.

Task 1.

• In order to analyze the function of two novel proteins in the GCN5 complex (CCDC101 and WDR18) that strongly repress PGC-1α glucose production function, we have designed and generated siRNAs to specifically knock-down these proteins.
• We have further tested the effects of knocking down these proteins using these siRNAs to demonstrate that they can regulate the transcriptional activity of PGC-1α in luciferase-based assays. CCDC101 and WDR18 are functioning as positive modulators of GCN5 Acetyl Transferase and therefore repress PGC-1α function.

Task 3.

• In order to identify the important functional acetylated lysines involved in PGC-1α repression, we have generated PGC-1α acetylated lysine mutants. PGC-1α R13 allele that contained mutated all the lysine identified by mass spectrometry was still acetylated by GCN5 Acetyltransferase.
• Consistent with the acetylation of the R13 mutant, GCN5 still repressed the transcriptional function of this PGC-1α allele.

Reportable Outcomes

Task 1 and Task 3. The results that are described in detail in this Research Technical Report have been presented in the following conferences or seminars. It is also expected that in the next year they will be send for publication in a peer-review journal.

- The Molecular biology of Obesity and Bone Remodeling. Cantoblanco Workshops on Biology. Madrid, Spain September, 2006.
- 8th Annual Conference. Frontiers in Diabetic Research. Molecular Mediators of Metabolic Homeostasis. Invited Speaker. Columbia University, College of Physicians and Surgeons. New York. November, 2006.
- 6th Nestle Nutrition Conference. Diet, genes and transcription factors linked to the Metabolic Syndrome. Invited Speaker. Mexico, November, 2006.
- University of Texas Health Science Center at San Antonio. Dept. of Physiology. Invited Speaker. San Antonio, TX. December, 2006.
- Keystone Symposia. Obesity: Peripheral and Central Pathways Regulating Energy Homeostasis. Invited Speaker. Keystone, Colorado, January 2007.

Conclusion

We have summarized in this Research Technical Report the experiments and results that have been obtained in this first year of this Award. Importantly, we have accomplished the tasks that were proposed in the original application. We have generated key reagents

(siRNAs for WDR-18 and CCDC110) that will allow us to accomplish the next tasks that are detailed in the Summary of Work. In addition, we have obtained important biological information identifying for the first time two proteins, WDR-18 and CCDC110, that are in the GCN5 complex and are regulating the transcriptional activity of PGC-1α. Our final goal is to determine the effects of these two proteins in blood glucose levels in several animal models as described in the application. Understanding the mechanism of action of this transcriptional regulatory complex that involves a chemical modification on PGC-1α will allow us to design specific drugs that can target the activities of these proteins and efficiently control blood glucose levels that are altered in metabolic diseases and situations of high stress.

References

1. Rodgers, J.T., *et al*. Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. *Nature* **434**, 113-118 (2005).
2. Lerin, C., *et al*. GCN5 acetyltransferase complex controls glucose metabolism through transcriptional repression of PGC-1alpha. *Cell Metab* **3**, 429-438 (2006).
3. Yoon, J.C., *et al*. Suppression of beta cell energy metabolism and insulin release by PGC-1alpha. *Dev Cell* **5**, 73-83 (2003).
4. Rhee, J., *et al*. Regulation of hepatic fasting response by PPARgamma coactivator-1alpha (PGC-1): requirement for hepatocyte nuclear factor 4alpha in gluconeogenesis. *Proc Natl Acad Sci U S A* **100**, 4012-4017 (2003).

Appendices

None.